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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 2861 for a patent by ES CELL INTERNATIONAL PTE LTD as filed on 07 June 2002.

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WITNESS my hand this
Ninth day of September 2003

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Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: SCREENING METHOD

Applicant: ES CELL INTERNTIONAL PTE LTD

The invention is described in the following statement:

SCREENING METHOD

FIELD

The present invention relates to methods of screening for compounds having an ability to modulate growth and/or differentiation of stem cells. More specifically, the invention relates to methods for the identification of compounds having an ability to prevent the differentiation of cultured human embryonic stem cells.

BACKGROUND

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo. ES cells may be derived from the inner cell mass of the blastocyst, which have the ability to differentiate into tissues representative of the three embryonic germ layers (mesoderm, ectoderm, endoderm), and into the extra-embryonic tissues that support development.

Because of their ability to differentiate into any cell type, the ability to culture ES cells *in vitro* is clearly desirable. However a significant problem is that ES cells tend to spontaneously differentiate in culture, leading to a loss in their pluripotency. This is undesirable as it would be beneficial to expand a population of hES cells without a loss in pluripotency.

This problem is conventionally addressed by using mammalian serum in culture systems for hES cells, and it has been shown that the removal of serum from ES cultures leads to a rapid loss of stem cell phenotype. However, serum contains a wide variety of biologically active substances which have the potential to adversely effect ES cell growth and differentiation. Furthermore, there is a biosafety issue if cells cultured in animal serum are subsequently used for implantation in a human or for the production of a biological therapeutic.

Other studies have employed defined media supplemented with FGF-2 to culture hES cells. However differentiation of ES cells under these conditions is more rapid than in the presence of serum, and animal cells are still required to maintain the hES cells.

Identification of compounds having an ability to inhibit differentiation will allow the design of simple culture media more suitable for human ES cell propagation. The prior art has not provided such a method to identify a satisfactory replacement for serum that allows for the culture of hES cells while inhibiting the differentiation of the cells

It is therefore an object of the present invention to at least alleviate a problem of the prior art.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method for identifying a compound capable of modulating the proliferation and/or differentiation of a stem cell, the method including the steps of exposing a stem cell having a G-protein coupled receptor to a test compound; and determining binding of the test compound to the receptor. Preferably, the G-protein coupled receptor is an endothelial differentiation gene receptor, and the stem cell is a human embryonic stem cell.

In another aspect the present invention provides a method for identifying a compound capable of modulating the proliferation and/or differentiation of a

stem cell, the method including the steps of exposing a stem cell having an extracellular-signal regulated kinase pathway; and determining activation of the extracellular-signal regulated kinase pathway. Preferably, the extracellular-signal regulated kinase pathway is a MAP kinase pathway and the stem cell is a human embryonic stem cell.

In a further aspect the present invention provides compounds capable of modulating the proliferation and/or differentiation of a stem cell identified by the methods described herein. Also provided is a cell culture medium including a compound identified by a method described herein.

In yet a further aspect the present invention provides a method for modulating the differentiation and/or proliferation of a stem cell having a G-Protein coupled receptor including the step of modulating the receptor. Preferably, the G-protein coupled receptor is an endothelial differentiation gene receptor, and the stem cell is a human embryonic stem cell.

A further aspect of the present invention provides a method for modulating the differentiation and/or proliferation of a stem cell having an extracellular signal regulated kinase pathway including the step of activating the pathway. Preferably, the extracellular-signal regulated kinase pathway is a MAP kinase pathway and the stem cell is a human embryonic stem cell.

DESCRIPTION OF THE FIGURES

FIGURE 1 shows Edg receptor mRNAs are expressed in HES cells. RT-PCR experiments were performed using mRNA isolated from HES cells using specific primers for human Edg receptors. In each case, experiments were conducted either in the presence (+) or absence (-) of reverse transcriptase. The RT-PCR products were separated by electrophoresis on 1.5% agarose gel and revealed by ethidium bromide fluorescence. Molecular sizes (in bp) of the products were calculated using 1 kB plus DNA ladder markers (M). These data are representative of at least 6 independent experiments, each carried out on mRNAs prepared from different cultures of HES cells.

FIGURE 2 shows S1P inhibits the spontaneous differentiation of HES cells. (A) HES cells grown with feeder, before the depletion in serum. (B) HES cells grown without serum after 8 days (B, C) and 12 days (D, E), in absence (B, D) or presence of S1P (C, E, 10 μM). These data are representative of at least 3 independent experiments.

FIGURE 3 shows S1P inhibits the spontaneous differentiation of HES cells. Double staining experiments were performed using antibodies for PCNA and GCTM-2. These data are representative of at least 3 independent experiments.

FIGURE 4 shows S1P stimulates ERKs phosphorylation in HES cells. Western blots experiments were performed using protein lysate from HES cells. (A) Cells were pre-treated or not with U0126 (30 μM, 1 hr) and incubated for 5 min in the absence (C, control) or presence of S1P (10 μM). (B) Cells were incubated for different time periods in the absence or presence of S1P (10 μM). (C) Cells were incubated for 5 min with various concentrations of S1P. The phosphorylation of Erk1 and Erk2 (P-Erk1 and P-Erk2) was assessed by immunoblotting with a polyclonal anti-active MAP kinase as described in Materials and Methods. After a stripping procedure, the same blots reprobed with a monoclonal anti-MAP kinase, allowed the detection of Erk1 and Erk2. These data are representative of at least 3 independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention provides a method for identifying a compound capable of modulating the proliferation and/or differentiation of a stem cell, the method including the steps of

exposing a stem cell having a G-protein coupled receptor to a test compound; and

determining binding of the test compound to the receptor.

Applicants have found that the modulation of the proliferation and/or differentiation of stem cells appears to be mediated by specific cell surface G-protein coupled receptors (GPCRs) called endothelial differentiation gene (Edg)

receptors. Accordingly, in a preferred method of the invention, the G-protein coupled receptor is an endothelial differentiation gene receptor.

To date, eight distinct mammal Edg receptors have been identified: Edg-1, Edg-2, Edg-3, Edg-4, Edg-5, Edg-6, Edg-7 and Edg-8. In a more highly preferred form of the invention the Edg receptor is selected from the group including: Edg-1, Edg-3, and Edg-5. Applicants have found that sphingosine-1-phosphate modulates Edg receptors 1, 3, and 5, resulting in an inhibition of differentiation of hES cells. Applicants have also found that this use of sphingosine-1-phosphate does not substantially negatively alter the ability of the stem cells to proliferate.

Preferably the stem cell is an embryonic stem cell. More preferably the stem cell is a human embryonic stem cell.

As used herein the term "embryonic stem cell" means a cultured cell line derived from preimplantation stages of development capable of differentiation into tissues representative of all three embryonic germ layers.

Theses cells express SSEA-3,SSEA-4, TRA 1-60, GCTM-2, alkaline phosphatase, Oct-4

- Grow as flat colonies with distinct cell borders
- Differentiate into derivatives of all three embryonic germ layers
- Feeder cell dependent (feeder cell effect on growth not reconstituted by conditioned medium from feeder cells or by feeder cell extracellular matrix)
- Highly sensitive to dissociation to single cells, poor cloning efficiency even on a feeder cell layer
- Do not respond to Leukemia Inhibitory Factor

In another aspect the present invention provides a cell culture medium substantially free of serum, the medium including a compound as identified by a method described herein.

The cell culture medium may be based on any of the base media known in the art useful for the growth and/or maintainance of hES cells. Such media include but are not limited to Dulbecco's Modified Eagles Medium (DMEM), KNOCKOUT-DMEM or HES medium. In a preferred form of the invention the medium is based on DMEM supplemented with insulin, transferrin and selenium.

The optimal concentration of a compound in the medium may be determined by routine experimentation. However, in a preferred form of the invention the phospholipid is present in the medium at a concentration of 0 to 20 μM In a highly preferred form of the invention the phospholipid is present in the medium at a concentration of 10μM. It would be expected that the optimum concentration will vary according to a number of parameters including the line of hES being cultured, the base medium used, and other culture conditions such as temperature, carbon dioxide concentration, and humidity.

In another aspect the present invention provides a human embryonic stem cell grown and/or maintained in a cell culture medium substantially free of serum, the medium including a compound identified by a method as described herein. Cells of the present invention will find many uses been devoted to the potential in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-basic embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation are based on the assumption that it will be possible to increase the proliferation and therefore grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation.

Applicants have used the screening assays as described herein to test the

ability of whether the lysophospholipids sphingosine-1-phosphate, LPA and C6-ceramide could modulate an extracellular-signal-regulated kinase, and therefore modulate the fate of hES cells in culture. Applicants show herein that hES cells are target cells for S1P and LPA, and that these cells express mRNA for the Edg receptors. Importantly the studies described herein show activation of ERK kinase is correlated with an inhibition of spontaneous differentiation of HES cells.

The invention will now be more fully described with reference to the following non-limiting Examples.

EXAMPLES

The following materials and methods were used in the Examples below.

Reagents

S1P AND LPA were obtained from Biomol (Plymouth Meeting, PA, USA) and were dissolved in methanol. Freshly prepared dilutions of agonists were made in water containing 0.1% fatty acid-free bovine serum albumin (BSA) (Sigma). Protease, sodium orthovanadate and U0126 were from Sigma. was from Calbiochem (San Diego, CA, USA). *Pertussis* Toxin (PTX) was from List Biological Laboratories (Campbell, CA, USA). GCTM-2, Oct-4, PCNA, Hoechst-33342

Cell culture

HES-3 cells were cultured as previously described Human stem cells were grown on MMC treated fibroblasts' feeder layer. Fibroblasts were plated on gelatine treated dishes. A combination of human and mouse derived fibroblasts were used at a density of approximately 25,000 and 70,000 cells per cm2 respectively. The fibroblasts were plated up to 48 hours before culture of the stem cells. Mouse fibroblasts only could also support the growth of the stem cells. However, while human fibroblasts could also support stem cells, they created an uneven and unstable feeder layer. Therefore, the human fibroblasts

were combined with the mouse fibroblasts to augment and achieve better support of growth and prevention of differentiation.

The medium that was used for the growth of human stem was DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS (Hyclone, Utah) (2-mercaptoethanol - 0.1mM (GIBCO), Non Essential Amino Acids - NEAA 1% (GIBCO), glutamine 2mM.(GIBCO), penicillin 50u/ml, and streptomycin 50mg/ml (GIBCO)

For direct observation, HES-3 cells were coated into 12-well plates (3 colonies per well), with or without mouse embryonic feeders (MEFs). The day following the plating, cells were incubated with the different agents in serum free medium containing insulin, transferring and selenium. Media was changed the 2nd day and then every 2 days.

For immunostaining, HES-3 cells were coated on chamber slides after mechanical dissociation, in order to obtain a monolayer culture. The day following the plating, cells were incubated with the different agents in a media depleted in serum. Media was changed the 2nd day and the cells were fixed 4 days after the first treatment.

For immunoblot analysis, cells were transferred into 24 well plates (8 colonies per well) without MEFs, and 24 hr later, were grown in the absence of serum for 18 hrs.

In some experiments, cells were pre-treated for 1 hr with U0126 (30 μM) or for 18 hrs with PTX (100 μg/ml).

RT-PCR experiments

Cells were washed with PBS and HES colonies were removed by treatment with protease. Purified mRNA was extracted from HES cultures using Dynabeads® Oligo $(dT)_{25}$ (Dynal, Oslo, Norway), according to the supplier's instruction. RT was performed using superscript™ II Rnase H$^-$ Reverse

Transcriptase (Invitrogen, Life technologies), according to the supplier's protocol. After cooling on ice, the cDNA samples were amplified by PCR with sense and antigens primers (synthesis performed by Sigma Genosys, Castle Hill, Australia) designed for the specific detection of human Edg-1, Edg-2, Edg-3, Edg-4, Edg-5, Edg-6, Edg-7 and Edg-8 DNA target sequences. The primers used for Edg-1, Edg-3, Edg-5, Edg-6 and Edg-8 were previously designed by Hornu□ *et al.* (2001) [1]. These primer pairs were : 5'-CCACAACGGGAGCAATAACT-3' (sense) and 5'-GTAAATGATGGGGTTGGTGC-3' (antigens) (expected PCR product: 480 bp) for Edg-1 ; 5'-TCAGGGAGGGCAGTATGTTC-3' (sense) and 5'-CTGAGCCTTGAAGAGGATGG-3' (antisense) (505 bp) for Edg-3 ; 5'-CCAATACCTTGCTCTCTCTGGC-3' (sense) and 5'-CAGAAGGAGGATGCTGAAGG-3' (antisense) (502 bp) for Edg-5 ; 5'-CGGCTCATTGTTCTGCACTA-3' (sense) and 5'-GATCATCAGCACCGTCTTCA-3' (antisense) (701 bp) for Edg-6 ; and 5'-TTCTGATACCAGAGTCCGGG-3' (sense) and 5'-CAAGGCCTACGTGCTCTTCT-3' (antisense) (460 bp) for Edg-8 . For Edg-2 and Edg-4, the primer pairs designed by Goetzl *et al.* (1999) were used: 5'-GCTCCACACACGGATGAGCAACC-3' (sens) and 5'-GTGGTCATTGCTGTGAACTCCAGC-3' (antisense) (621 bp) for Edg-2, and 5'-AGCTGCACAGCCGCCTGCCCCGT-3' (sense) and 5'-TGCTGTGCCATGCCAGACCTTGTC-3' (antisense) (775 bp) for Edg-4. For Edg-7, the primer pairs designed by Goetlz *et al.* (2000) were used: 5'-CCATAGCAACCTGACCAAAAAGAG-3' (sense) and 5'-TCCTTGTAGGAGTAGATGATGGGG-3' (antisense) (482 bp). PCR experiments were performed in a mixture (25 µl) containing 0.25 units of Taq DNA polymerase (Biotech International Ltd, Perth, WA, Australia) and 2 µM of each primer in a buffer including 67 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.25 mM of each dATP, dGTP, dCTP, dTTP. Absence of contaminating genomic DNA was confirmed by control reactions with mRNA that had not been treated with reverse transcriptase. PCR experiments were run with the following steps: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C (Edg-1, Edg-3, Edg-5, Edg-6, Edg-8) or 56°C (Edg-2, Edg-4, Edg-7) for 2 min,

extension at 74°C for 2 min, and a final extension at 74°C for 7 min. The specific amplified DNA fragments were purified by electrophoresis on 1.5 % (w/v) agarose gel, stained with ethidium bromide and photographed. The amplicons were purified and sequenced.

Immunofluorescence

Cells were washed in PBS, fixed with MeOH, and immunostaining was performed, using the specific stem cell marker antibody GCTM-2, and the specific cell proliferation marker PCNA. Cells were then washed and the nucleuses were stained with Hoechst-33342 (1□g/ml). Slides were mounted and then observed by fluorescent microscopy. Cells were then counted in order to determine the ratio of proliferating stem cells within the overall population.

Western blot analysis

HES3 cells were lysed following removal of the supernatants by addition of a reducing loading buffer (2% SDS, 62.5 mM Tris pH 6.8, 0.1 M DTT, 0.01% bromophenol blue) containing 1 mM sodium orthovanadate. Samples were boiled for 10 min and centrifuged at 13000g for 15 min, and protein lysates (approx. 80 µg) were separated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide, w/v). Proteins were transferred to nitrocellulose (Hybond-ECL, Amersham) and immunoblotting was carried out with rabbit polyclonal anti-active mitogen-activated protein (MAPK) antibodies raised against a dually phosphorylated MAPK peptide (Promega, Madison, WI, USA). Peroxidase-coupled secondary antibody (Dako) was detected by exposure of autoradiographic films in the presence of a chemiluminescent detection reagent (ECL, Amersham). Stripping of antibodies was achieved by incubating the membrane during 30 min at 50°C in a buffer containing 100 mM □mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7. The membrane was then reprobing with rabbit polyclonal anti-ERK1/2 antibodies (Promega), and then with peroxidase-coupled secondary antibodies (Dako).

Blots probed with either rabbit polyclonal anti-active p38 (Promega) or rabbit polyclonal anti-active JNK (Promega) or mouse polyclonal GCTM-2 antibodies

were also performed, using the same procedure as described above.

Protein quantification

HES3 cells were lysed and their quantity was determined by using a colorimetric assay based on the Bradford dye-binding test (Bio-Rad Laboratories, Regents Park, NSW, Australia).

Each set of experiments was performed at least 3 times (*n* refers to number of independent experiments performed on different cell cultures).

EXAMPLE 1: Edg mRNAs are expressed by HES cells

The results presented in Figure 1A indicate that HES cells expressed mRNA transcripts for the three S1P receptors : Edg-1, Edg-3 and Edg-5 while these cells do not seem to express mRNA for Edg-6 and Edg-8 (data not shown). Moreover, HES cells express mRNA transcripts for each of LPA receptors : Edg-2, Edg-4 and Edg-7 (Figure 1B). The nucleotide sequences of all purified PCR products were analysed and revealed to be identical to the corresponding regions in the human receptor genes.

EXAMPLE 2: S1P prevents HES cells from spontaneous differentiation

Applicants next determined whether S1P could modulate the fate of HES cells. When HES cells were grown on MEFs, in a culture media depleted in serum, they spontaneously differentiated. As shown in Figure 2, after 8 days in such conditions, HES cells colonies contained enlarged flattened cells which formed cystic structures (Figure 2A, 2B). Even after 12 days, LPA (up to 50 μM) did not seem to affect the growth of the colonies (data not shown). In presence of S1P (10 □M, 8 days), the colonies were more compact and less differentiated than in the control condition (Figure 2C). This effect of S1P was more obvious after 12 days of treatment (Figure 2D, 2E). The inhibitory effect of S1P on cell differentiation and the lack of effect of LPA were also observed when HES cells were grown without MEFs, suggesting that S1P did not directly act on the feeder cells (n=3, data not shown).

In order to understand and quantify the effect of S1P on the spontaneous differentiation of HES cells, double immunostaining experiments were carried out. For that purpose, Applicants used two specific antibodies, one as a stem cell marker, GCTM-2, and one for proliferation, PCNA, a marker that is only expressed during the S phase of the cell cycle, in order to determine the ratio of proliferating stem cells (Figure 3). After 4 days in a media without serum, most of the control cells were differentiated (Figures 3A, 3C and 3E), as revealed by the fact that only 16± % of the cells still expressed GCTM-2 (Figure 4A). By contrast, when S1P (10 μM) was added to the media, 68± % of the cells were GCTM-2 positive, suggesting that most of the cells remained stem cells (Figures 3B, 3D, 3F and 4B). Within these cell populations, a large part expressed PCNA, suggesting that most of these stem cells still proliferated (Figures 3G and 3H). However, no marked difference in the proliferating rate of HES cells between the control cells and the ones treated with S1P were observed (Figure 4). Altogether, these data suggest that S1P mostly acts on the differentiation of HES cells observed in absence of serum rather then acts on the proliferating state of HES cells.

EXAMPLE 3: S1P activate ERKs in HES cells

Because the MAP kinases ERKs have often been implicated in cell proliferation and differentiation, the effects of S1P on the activation of the ERKs were then investigated. After 5 min, S1P stimulated the phosphorylation of ERKs in HES cells (Figure 4), an effect that was totally inhibited in presence of the MEK inhibitor U0126 (30 μM) (Figure 4A). S1P stimulated ERKs for at least 60 min and in a concentration dependant manner (Figure 4B, 4C).

These results show clearly that treatment of human ES cells with S1P results in inhibition of spontaneous differentiation. S1P is a major component of serum, and is therefore likely to account for much of the beneficial effect of calf serum in human ES cultures. Although human ES cells express receptors for both S1P and LPA, the latter lysophospholipid is inactive on human ES cells. This

suggests that particular members of the Edg receptor family have distinct effects on human ES cell behaviour.

REFERENCES

Hornuss, C., et al., Human and rat alveolar macrophages express multiple EDG receptors. Eur J Pharmacol, 2001. **429**(1-3): p. 303-8.

Goetzl, E.J., et al., Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res, 1999. **59**(18): p. 4732-7.

Goetzl, E.J., Y. Kong, and J.K. Voice, Cutting edge: differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. J Immunol, 2000. **164**(10): p. 4996-9.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 7 June 2002
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ES CELL INTERNATIONAL PTE LTD

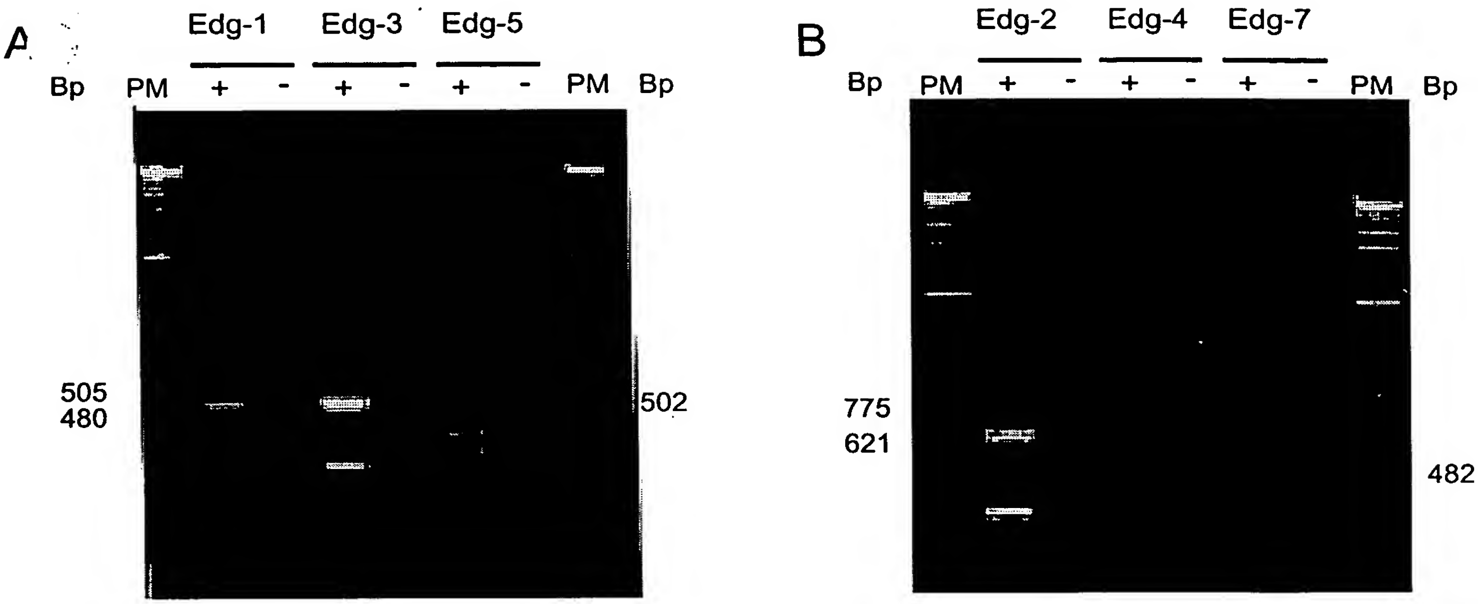
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FIGURE 1

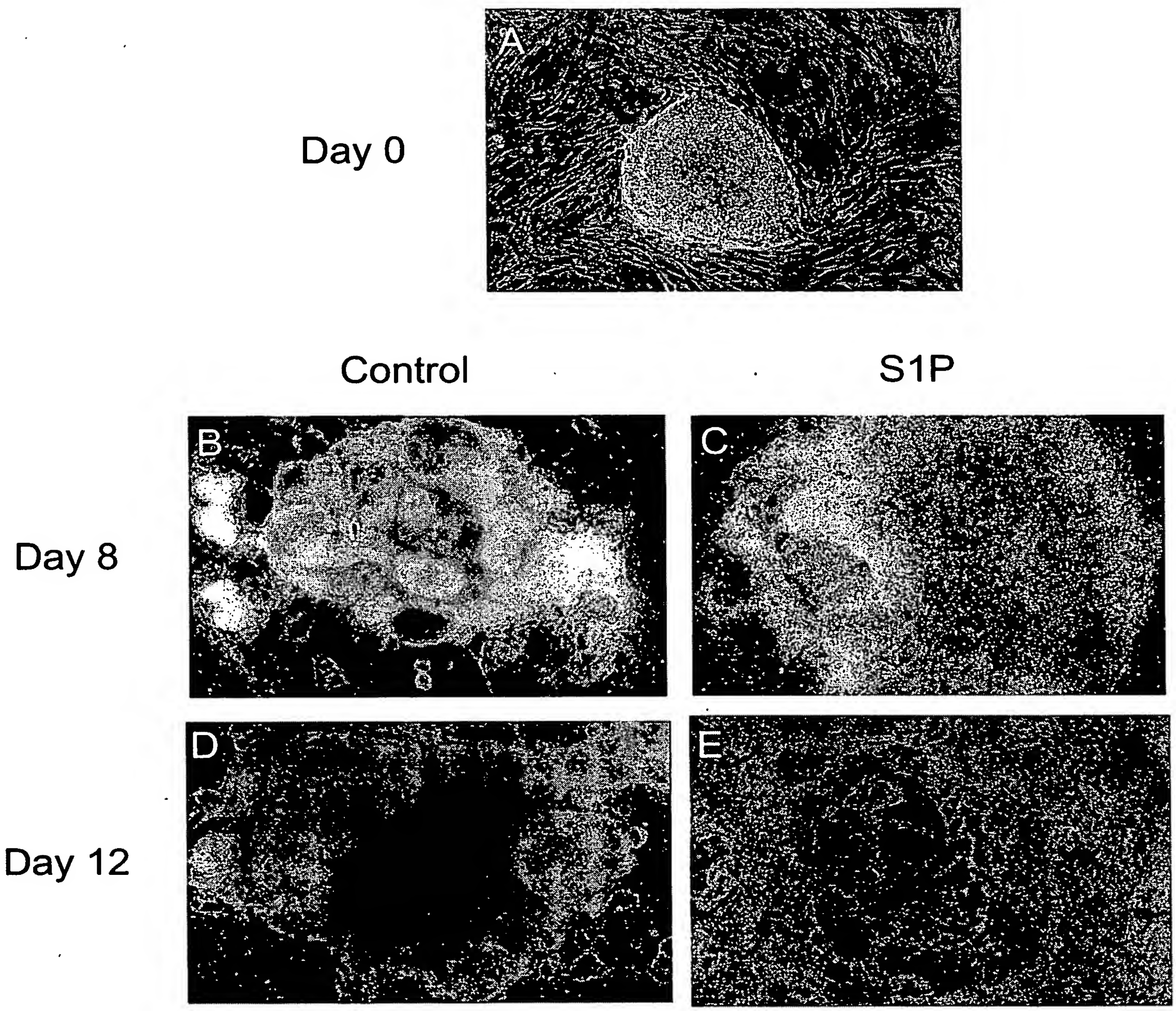


FIGURE 2

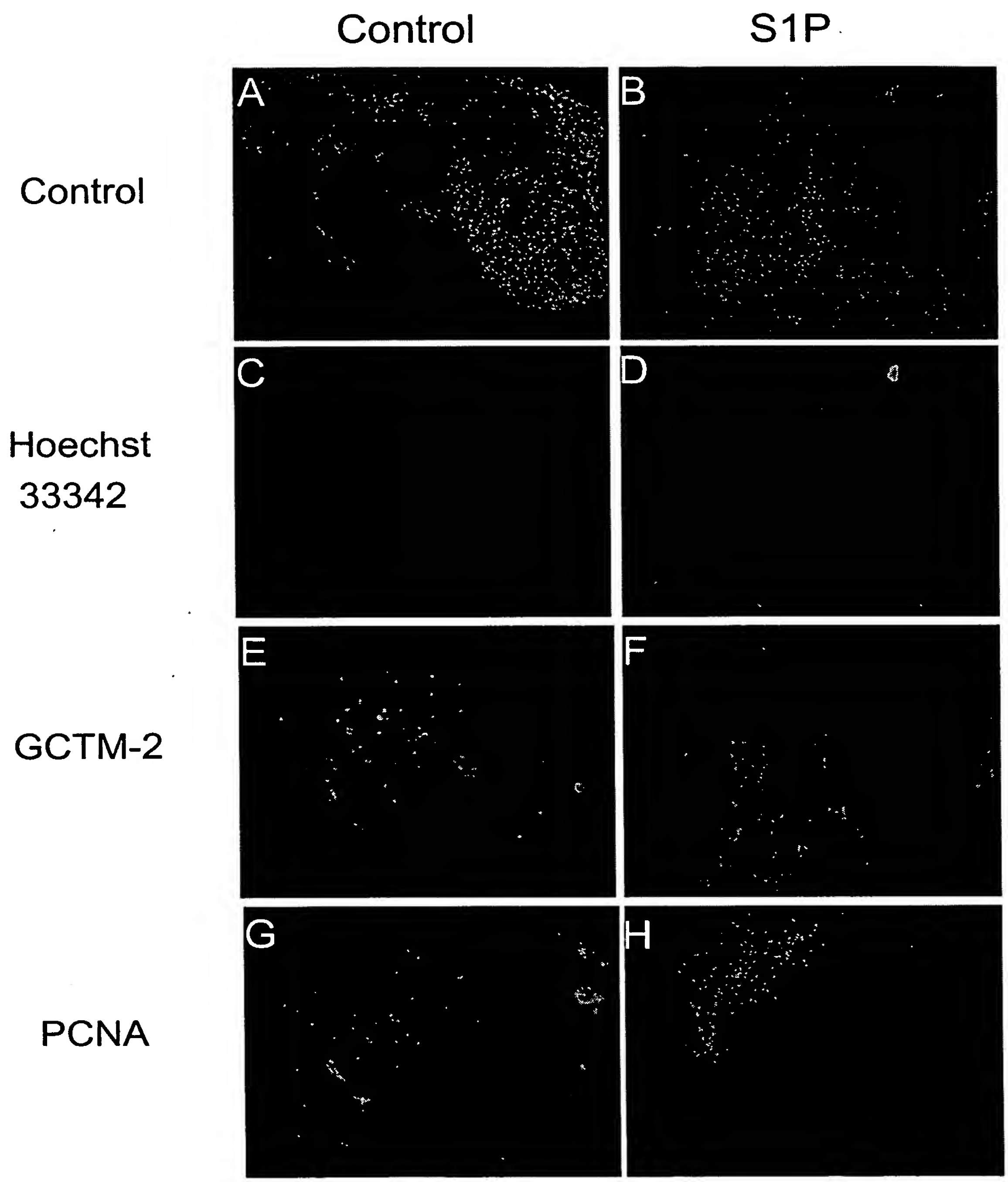
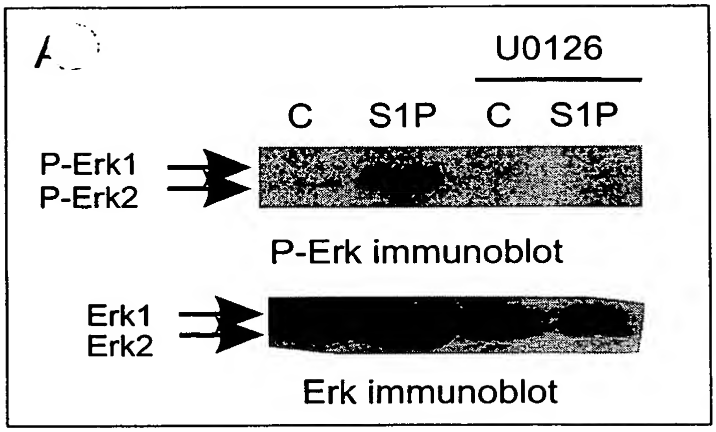


FIGURE 3


A
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C
S1P
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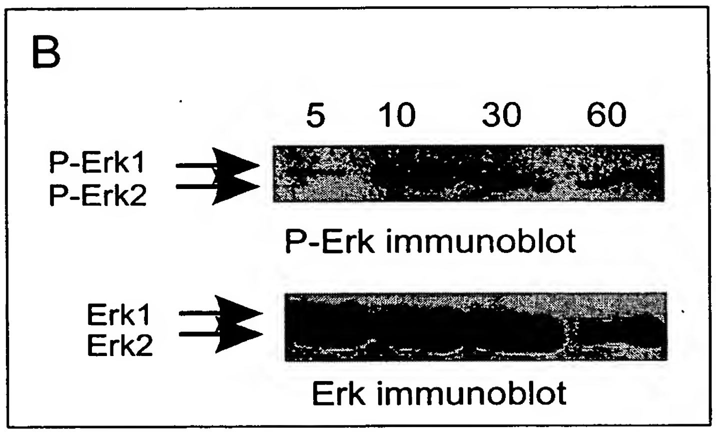
Erk1
Erk2
Erk immunoblot

FIGURE 4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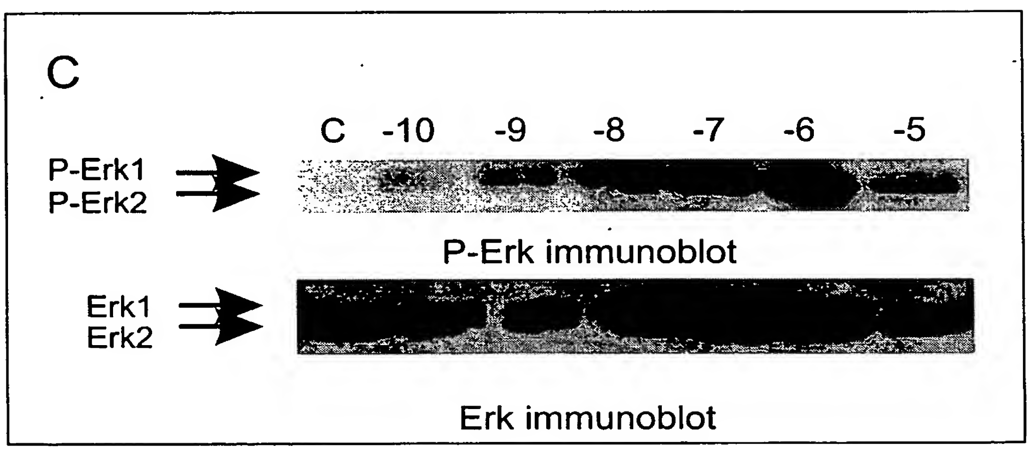
Erk1
Erk2
Erk immunoblot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